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Original Articles

Fungal assemblages in predictive stream bioassessment: A cross-taxon comparison along multiple stressor gradients

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ABSTRACT

Degradation of freshwater ecosystems requires efficient tools for assessing the ecological status of freshwater biota and identifying potential cause(s) for their biological degradation. While diatoms and macroinvertebrates are widely used in stream bioassessment, the potential utility of microbial communities has not been fully harnessed. Using data from 113 Finnish streams, we assessed the performance of aquatic leaf-associated fungal decomposers, relative to benthic macroinvertebrates and diatoms, in modelling-based bioassessment. We built multi-taxon niche -type predictive models for fungal assemblages by using genus-based and sequence-based identification levels. We then compared the models' precision and accuracy in the prediction of reference conditions (number of native taxa) to corresponding models for macroinvertebrates and diatoms. Genus-based fungal model nearly equalled the accuracy and precision of our best model (macroinvertebrates), whereas the sequence-based model was less accurate and tended to overestimate the number of taxa. However, when the models were applied to streams disturbed by anthropogenic stressors (nutrient enrichment, sedimentation and acidification), alone or in combination, the sequence-based fungal assemblages were more sensitive than other taxonomic groups, especially when multiple stressors were present. Microbial leaf decomposition rates were elevated in sediment-stressed streams whereas decomposition attributable to leaf-shredding macroinvertebrates was accelerated by nutrients and decelerated by sedimentation. Comparison of leaf decomposition results to model output suggested that leaf decomposition rates do not detect effectively the presence of multiple simultaneous disturbances. The rapid development of global microbial database may soon enable species-level identification of leaf-associated fungi, facilitating a more precise and accurate modelling of reference conditions in streams using fungal communities. This development, combined with the sensitivity of aquatic fungi in detecting the presence of multiple human disturbances, makes leaf-associated fungal assemblages an indispensable addition in a stream ecologist's toolbox.

1. Introduction

Many legislative mandates, such as the Water Framework Directive in Europe or Clear Water Act in USA, have been launched to foster the protection and management of surface waters. These approaches are usually based on the use of minimally disturbed reference conditions as a benchmark for the biological condition of aquatic ecosystems (Stoddard et al., 2006). The standard tool for predictive reference condition-based bioassessment is the multitaxon niche modelling approach (such as RIVPACS; Moss et al., 1987), which typically uses environmental attributes insensitive to human influence to predict the number of native taxa expected in the absence of human disturbance. The biological impairment is quantified by the O/E ratio, which is the relative difference between the observed (O) and expected (E) number of taxa (Hawkins, 2006). O/E quantifies the integrity of native biota and is thus a globally consistent and ecologically meaningful measure of the biotic condition of a site. The multitaxon niche modelling approach was initially developed for lotic macroinvertebrates (Moss et al., 1987) but has been adopted also for many other taxonomic groups, including riverine fishes (e.g. Joy and Death, 2002), macrophytes (Jyväsjärvi

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et al., 2014), diatoms (Feio et al., 2007) and lake biota (e.g. Jyväsjärvi et al., 2011).

Recent innovations in molecular biology and next-generation DNA sequencing techniques (Knight et al., 2018) have increased the technological capabilities of sequencing and the cost-efficiency and comprehensiveness of the identification of terrestrial and aquatic microbiota (Seena et al., 2010). However, despite growing interest in using microbes in freshwater and marine biomonitoring (Emilson et al., 2016; Goodwin et al., 2017), microbial assemblages are underutilized in stream bioassessment and to date, no attempt has been made to evaluate their suitability for the reference condition-based biological assessment of freshwater ecosystems. Here, we assessed the modelling-based performance of aquatic leaf-associated fungi relative to more conventionally used diatoms and benthic macroinvertebrates in detecting the effects of anthropogenic stressors on stream ecosystem integrity.

Riparian leaf litter plays a fundamental role in many stream ecosystems, providing a basal carbon and energy source for a vast number of organisms. Given the focal role of leaf decomposition in stream ecosystem functioning, it has been repeatedly advocated as a universal measure of stream health (Gessner and Chauvet, 2002, Dangles et al., 2004, Chauvet et al., 2016). Studies testing this idea have yielded variable results, however: for instance, nutrient enrichment often enhances decomposition (Webster and Benfield, 1986; Suberkropp and Chauvet, 1995; Woodward et al., 2012; Jyväsjärvi et al., 2020), whereas acidification tends to decelerate decomposition rates (Mulholland et al., 1987; Dangles et al., 2004). Even studies manipulating the same environmental stressor have produced different, and sometimes even opposing results. For example, while Matthaei et al. (2010) reported sedimentation to enhance leaf decomposition, Mustonen et al. (2016) found greatly reduced rates in their experiment. The response of leaf decomposition to nutrient enrichment is known to be hump-shaped (Woodward et al., 2012), but it also depends on the environmental context and type of leaf species (Ferreira et al., 2015). Such a high variability in stressor responses challenges the utility of leaf decomposition as an integrated measure of stream health, particularly when multiple anthropogenic stressors act in concert (Piggott et al., 2015a).

Despite the often strong coupling of structure and function (e.g. Bell et al., 2005), these two elements of the decomposition process do not always show parallel responses to human disturbance. While some studies have shown substantial variation in leaf decomposition rates with no concurrent changes in biological communities (e.g. McKie and Malmqvist, 2009), there is also evidence that anthropogenic stress may cause marked structural changes that do not translate to altered decomposition rates (Bärlocher and Graça, 2002; Dang et al., 2005). This implies a high degree of functional redundancy in decomposer communities; human disturbance may then be first detected at the assemblage level while the process of decomposition may only be altered after a more pronounced abundance change of the functionally most important species (Pascoal et al., 2005a, 2005b).

Most approaches to bioassessment focus on single-stressor situations, while in reality, ecosystems face multiple simultaneously operating stressors; the detection of such multiple-stressor effects is a key challenge to environmental management. By using data from near-natural reference streams, we developed multitaxon niche models for macroinvertebrates and diatoms, and for leaf-associated fungal assemblages using both genus-level blasted data and sequence-based OTU (operational taxonomic unit) data. The models were then applied to a set of non-reference streams disturbed by either nutrient enrichment, acidification, sedimentation, or combinations of the three (nutrients + sedimentation; nutrients + acidification). We also measured alder leaf decomposition rate at a subset of the sites to compare assessment outputs between the modelling-based approach and a key measure of stream ecosystem functioning. Given the great species diversity of leaf-associated fungal communities and their known sensitivity to human-induced stressors, we expected fungi-based models to be at least equally good as, or even more precise and sensitive than, those based on more traditional biological target groups of bioassessment. We further expected that, compared to diatoms and macroinvertebrates, fungal communities should respond more coherently and predictably to several interactive stressors (additive responses), thereby reducing the likelihood of 'ecological surprises' (Paine et al., 1998). We further expected multiple, simultaneously operating stressors to have complex interactive effects on most biological responses, with fungi potentially showing the strongest responses of the investigated taxonomic groups (see Tolkkinen et al., 2013). Finally, we expected that the structure of leaf-associated fungal communities, rather than the associated ecosystem function (i.e. leaf decomposition), is more likely to allow the detection of status impairment, especially in multiple-stressor situations where different stressors may have different and even opposing effects on decomposition rate.

2. Material and methods

2.1. Study sites

We compiled biological and environmental datasets maintained by Finnish Environment Institute and University of Oulu for 113 streams. Our study sites are located in central Finland (62–66°N and 21–29°E; Fig. 1) in an area characterized by peatlands and mixed forests. Streams in the area are typically slightly acidic and colored by humic substances.

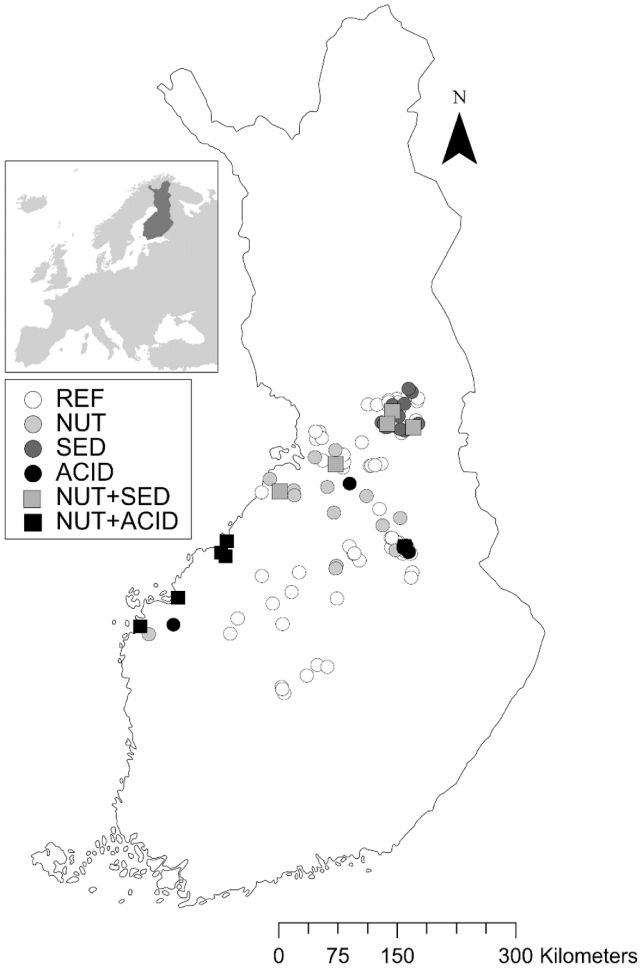

Fig. 1. Location of the study sites in central Finland. Different symbols represent different stressor types. REF = Reference sites; test sites are grouped by the main stressor type: SED = sedimentation; NUT = nutrient enrichment; ACID = acidification; NUT + SED = nutrients and sedimentation; NUT + ACID = nutrients and acidification.

The main anthropogenic pressures in the area are i) agricultural diffuse and point-source nutrient enrichment, ii) forestry-induced land drainage that causes increased streambed sedimentation, and iii) acidification. The study sites range from 1st to 4th order streams and sampling sites consist of riffles about 20 × stream width. Based on available water chemistry data and expert judgement of dominant land-uses, 67 sites were selected to represent near-pristine reference sites (hereafter 'REF' sites). The remaining 46 sites were assigned into groups according to the main environmental stressor(s) present: sites degraded mainly by nutrient enrichment (hereafter, NUT; n = 14), sedimentation (SED; n = 11), acidification (ACID, n = 10), and multi-stressed sites degraded by both NUT and SED (n = 6) or NUT and ACID (n = 5) (Fig. 2).

2.2. Diatom and macroinvertebrate sampling

Diatoms and macroinvertebrates were sampled between 2001 and 2015 during September-October by trained personnel using national guidelines (Meissner et al., 2016). Diatoms were sampled from the upper surfaces of five randomly selected cobble-sized (Ø 10–15 cm) stones at each site. The biofilm on upper stone surfaces was washed with water into a 50–100 ml jar using a toothbrush, and the dislodged material was preserved by adding ethanol to reach a concentration of 20% by volume. From each sample, 400 diatom valves were counted and identified to species, species group or genus level. Benthic macroinvertebrates were sampled by taking four 30-s kick-net (mesh size 500 µm, moving distance 1 m) samples covering most microhabitats present at a site. This method is known to capture about 75% of taxa present in a given reach, mainly missing species with sporadic occurrence in streams (Mykrä et al., 2006). Invertebrates were preserved in 70% ethanol in the field and samples were later sorted in the laboratory. All invertebrates except oligochaetes and chironomids were identified and counted, mainly to species or genus.

2.3. Leaf decomposition assays and fungal assemblages

Leaf decomposition rates were measured in 2009–2015 using litter bags. In most cases (76%), the sampling year corresponded with other biological monitoring (see above). For some (24%) sites, the sampling years differed (1–14 yr), but comparison of available water quality and land-use data showed negligible alteration of catchment area/stream conditions between the time periods. Leaf decomposition was measured using two different mesh sizes; fine mesh bags (0.2 mm) were used to measure microbial decomposition, while coarse mesh bags (8 mm) allowed invertebrates to enter and were thus used to measure shredder-mediated decomposition (see below). Four grams of dried alder (*Alnus incana*) leaves were enclosed in 15 × 15 cm mesh bags. Four bags of each mesh size were anchored onto the stream bed with house bricks and cable ties in early September. The bags were removed after 30 days and transferred to a laboratory freezer (−20 °C). In the laboratory, litter bags were gently cleaned with a spray bottle and tap water to remove any accumulated sediments, twigs, needles and macroinvertebrates. The remaining leaf material was dried for 48 h at 60 °C, weighed, ashed for 4 h at 550 °C and reweighed to estimate ash-free dry mass (AFDM). Leaf breakdown rates (k) were determined using the negative exponential decay model (Benfield, 1996, see also Lecerf and Chauvet, 2008) using a set of 10 unincubated leaf bags of both mesh sizes as a baseline. Decomposition rate in coarse bags was calculated as coarse minus fine-mesh bags, representing decomposition caused mainly by macroinvertebrate feeding. We used temperature loggers (iButton; Thermochron, Maxim Integrated, San Jose, USA) to measure water temperature at 30-min intervals and decomposition rates were corrected for degree days by replacing time in the exponential model by the cumulative daily mean temperature. However, water temperature data were lacking for 15 REF sites, two NUT and SED sites, four ACID sites and one NUT + SED and NUT + ACID sites (25 sites in total) and thus only 88 sites were used for the analysis of decomposition rates.

Leaf-associated fungal assemblages were identified from the fine mesh bag leaf samples. A subsample (0.07 g) of frozen leaf material was taken from each bag for the extraction of fungal DNA. Fungal assemblage composition was determined either by 454 pyrosequencing (34% of the samples) or IonTorrent (76%). The protocol for sequence data harmonization is specified below. Subsamples of frozen leaf material were freeze-dried and pulverized and fungal DNA was extracted using PowerSoil DNA Isolation Kit (MO BIO laboratories, Carlsbad, California, USA). Each fungal sample was diluted to 5 ng μl^{-1}. Fungal rRNA coding for both methods was amplified using ITS primer ITS1F 5′-CTTGGTCATTTAGAGGAAGTAA-3′, while R-primers 58A2R 5′-CTGCGTTCTTCATCGAT-3′ and ITS4R 5′-TCCTCCGCTTATTGATATGC-3′ were used for Ion Torrent and 454 system, respectively (Martin and Rygiewicz 2005). Triplicate 20-µl PCR reactions contained 10 ng of template DNA, 1× Phusion HF buffer, 0.2 µM of forward and reverse primers, 0.2 mM dNTP's and 0.4 U of Phusion high-fidelity DNA polymerase, the Ion Torrent reactions being amplified using the protocol by Lehosmaa et al. (2018) and the 454 reactions by Tolkkinen et al. (2013).

The amplicons were sequenced using the GS FLX 454 system (Roche, Basel, Switzerland) (n = 43) or the Ion Torrent PGM sequencer (n = 70) with Ion HiQ chemistry and 316 chips at Biocenter Oulu Sequencing Center (University of Oulu, Finland). Prior to further analysis, reads from the 454 run were trimmed to 58A2F primer sequence 5′-ATCGATGAAGAACGCAG-3′ with cupadapt program (Martin, 2011). The

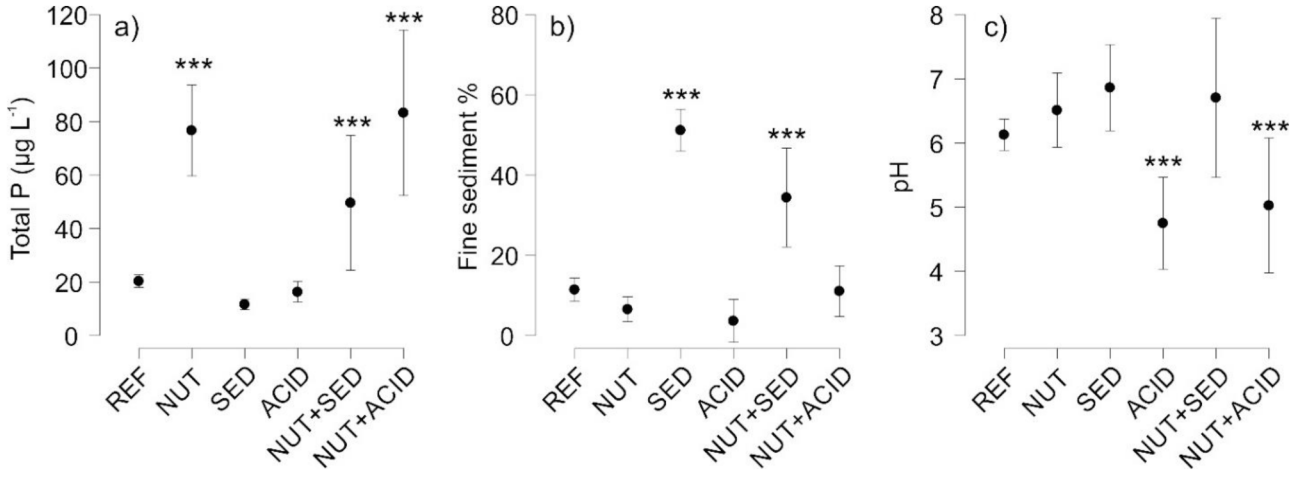

Fig. 2. Mean (±95 CI) a) total phosphorus concentration, b) percent cover of fine (<2 mm) sediments and c) water pH in each stream group. Statistical significance relative to REF sites (GLM) is denoted by asterisks (***p < 0.001). For stream-type acronyms, see Fig. 1.

trimming step resulted in sequences with a similar length from the ITS1 region for both NGS methods (Martin and Rygiewicz, 2005). The sequences were analyzed and combined using Quantitative Insights into Microbial Ecology (QIIME) pipeline (Caporaso et al., 2010). The sequence library was split by samples and quality-filtered based on the quality scores for every sequence. Quality scores below 20 were removed, and the minimum and maximum sequence lengths were 200 bp and 1000 bp, respectively. Sequences with ambiguity and more than two mismatches in the primer were also removed. Sequences were clustered into operational taxonomic units (OTUs) using the *Usearch61* algorithm, clustering OTUs at 97% identity against UNITE ITS database (Edgar, 2010; Kõljalg et al., 2013), and chimeric OTUs were detected using UCHIME method (Edgar et al., 2011).

We analyzed separately OTUs based on DNA sequence-similarity (hereafter, sequence OTUs) and OTUs assigned to genus-level using BLAST (basic local alignment search tool) UNITE ITS database. As sequence numbers varied among the samples, both sequence and blasted-based data were rarefied to the lowest shared sample size (n = 932) and singletons were discarded prior to analyses. The initial number of fungal OTUs was 9885. Our early attempts to include all sequence OTUs resulted in inaccurate models, and we therefore excluded the rarest (occurring < 5% of the sites) OTUs from the sequence-based fungal data.

2.4. Environmental data

A wide array of environmental variables was measured *in situ* (simultaneously with biological sampling) or later in GIS and used either for calibration of the multi-taxon niche models or classification of non-reference sites. The percentage cover of different substrate types were visually determined in ten 0.25 m^2 plots using a modified Wentworth scale from 1 (clay/silt 0.001–0.07 mm) to 10 (large boulder to bedrock >512 mm). Current velocities (m s^{-1}) were measured from the same plots simultaneously with biological sampling using Schiltknecht® MiniAir20. Water samples were analysed for total phosphorus, total nitrogen, water colour and pH following national standards (National Board of Waters, 1981). We also measured several variables describing land cover (e.g. % lakes; see Table S1) and land use (e.g. % agriculture, % urban) properties from national GIS-databases using ArcMap Desktop 10.5 (ESRI, 2017). Climate data were obtained from the WorldClim dataset (version 1.4; Hijmans et al., 2005, http://www.worldclim.org/) at 30 arcsec resolution.

2.5. Development of predictive models

We compiled data from 67 REF streams to develop RIVPACS -type multitaxon niche models for macroinvertebrates, diatoms, genus-level fungal data and sequence-based fungal OTU data. First, for each taxonomic group, we clustered the REF sites according to their biological similarity using an agglomerative hierarchical flexible-β clustering algorithm ($\beta = 0.6$) with the Sørensen dissimilarity measure for presence/absence data. We then truncated each dendrogram to produce six site groups with at least five REF sites (see Aroviita et al., 2009; Jyväsjärvi et al., 2011). The biological dissimilarity of the resulting clusters was visualized using a non-metric multidimensional scaling (NMDS) based on Sørensen dissimilarity -based metaMDS function of vegan R package (Oksanen et al., 2019). We used Random Forests (RF) models (Breiman, 2001) to predict the probability for a site to belong to each of the biologically defined groups as a function of environmental variables insensitive to human activity. For the final RF model, we aimed to find a parsimonious set of important variables for prediction using a VSURF R package (see Genuer et al., 2015). We applied the RF models to predict the probability of cluster membership (G_j) for all study sites (j). We then calculated probability to capture (p) each taxon (i) in each site in reference conditions following the RIVPACS protocol (e.g. Moss et al., 1987). Expected number of taxa (E) was obtained by summing the product of site probabilities (G_j) and taxon probabilities (p_{ij}), calculated as frequencies of taxa occurrence among clusters:

$$E = \sum_{j=1} G_j p_{ij}$$

We then calculated the number of predicted taxa that were also observed (hereafter O) and related O with E to obtain the O/E-taxa ratio. O/E ratio close to 1.0 indicates high biological integrity of a site, whereas values < 1.0 suggest that the site has lost taxa relative to its reference-condition expectation. Taxa with small capture probabilities ($p < 0.5$) are commonly excluded from macroinvertebrate-based O/E calculations because rare taxa tend to decrease model accuracy and precision (Hawkins et al., 2000). However, as the optimal p threshold for fungal assemblages was not known *a priori*, we calculated O/E ratios for all biological groups using 10 p values ranging from 0 to 0.5 with 0.05 increments. We then used the average across these values as the main model output. This guarantees equal treatment of all taxonomic groups regardless of taxonomic richness.

In addition to traditional O/E ratios (hereafter O/E_{TAXA}), we calculated the BC-index of Van Sickle (2008). The BC index summarizes the taxon-specific disparity between observed and expected assemblages, including both the loss of expected and occurrence of unexpected taxa. All taxa are used for the calculation of BC. Thus, BC considers the occurrence of rare taxa and is more suitable as an assessment metric for the entire community. BC indices were calculated using all species (i.e. $p = 0$) and sites as follows:

$$BC = \frac{\sum |O_i - p_i|}{\sum (O_i + p_i)}$$

where O is the observed occurrence and p is the predicted probability of capture of taxon i. To render BC-index comparable to O/E_{TAXA}, we converted the index values to similarities by subtracting dissimilarities from one and then scaling the values (hereafter O/E_{BC}) by dividing them with the average BC-1 of the reference sites (see Rääpysjärvi et al., 2016).

2.6. Model evaluation

Given the limited number of REF sites, we were unable to validate our models with independent data. Instead, we used leave-one-out cross-validation to evaluate the models. Cross-validation was done by estimating the E value for each REF site by excluding that particular site from the REF set in calculation of taxon probabilities while keeping the RF model constant for site probabilities (see Aroviita et al., 2009). To summarize model *accuracy*, we calculated the average cross-validated O/E_{TAXA}-ratios for the REF sites, whereas *precision* was measured with standard deviation of REF O/E_{TAXA} ratios. Coefficients of determination (R^2) and normalized root mean squared errors (NRMSE) of E and O were also used to summarize model performance. For an accurate and precise model, mean O/E_{TAXA} among REF sites should be close to one and standard deviation should be low, and the expected number of taxa should correspond to that observed (high R^2 and low NRMSE). We evaluated the performance baseline of our models by using null models where all sites are allocated to one group, yielding a single E for all sites (Van Sickle et al., 2005).

2.7. Other statistical methods

We used generalized linear models (GLM) to test for differences in O/E_{TAXA} and O/E_{BC} values between reference sites and the five disturbed stream groups (NUT, SED, ACID, NUT + SED, NUT + ACID). Similar analyses were also conducted for leaf decomposition rates. To improve normality and homoscedasticity, we applied box-cox transformations for the response variables. We then performed two-way ANOVAs (additive model) separately for O/E_{TAXA} and O/E_{BC} ratios for each stressor

combination (NUT × SED; NUT × ACID) to test whether the stressors had any indirect (i.e. antagonistic or synergistic) effects on the response variables. For interpretation, we used the directional classification system of Piggott et al. (2015b) which combines the magnitude and direction of the cumulative response and the interaction effect (effect of deviation from the additive model prediction) to determine synergism and antagonism relative to individual stressors.

3. Results

3.1. Development of the predictive models

The macroinvertebrate data comprised 109 taxa and the diatom data 370 taxa. The genus-based fungal richness was 378, whereas taxa richness of the sequence-based fungal data was substantially higher (5202 OTUs). Based of BLAST hits, leaf-associated fungal communities were dominated by Ascomycota (60%), Basidiomycota (32%), Chytridiomycota (5%), Glomeromycota and Zygomycota (~3%). Phylum Ascomycota is mostly comprised of aquatic hyphomycetes which are the predominant leaf-decomposing fungal group in stream ecosystems and hyphomycetes *Fusarium* sp. and *Articulospora* sp. were common fungi in our data. Fungal communities were also characterised by foliar tree host-associated microfungi such as genus *Mycosphaerella*, which have been previously identified from leaf litter in boreal stream ecosystems (Koivusaari et al., 2019).

Clustering of macroinvertebrate assemblages (Fig. 3a) was best explained by stream width, catchment size and proportion of lakes in the catchment (Table S1). Classification error rate for the macroinvertebrate random forest (RF) model was 32.8%. Diatom clustering (Fig. 3b; RF error rate 34.3%) was explained by current velocity, mean temperature of the warmest quarter, stream width and proportion of lakes (Table S1). Clustering of leaf-associated fungal genus-level data (Fig. 3c; RF error rate 31.3%) was explained by current velocity, proportion of lakes and stream width and depth (Table S1). Sequence-based fungal clustering (Fig. 3d; RF error rate 40.3%) was explained by current velocity, catchment size and minimum temperature of the warmest quarter (Table S1).

Mean expected numbers of taxa (E) among the 67 REF sites ranged from 19.8 to 78.6, being lowest for diatoms and highest for sequence-based fungi. For all taxonomic groups, the crossvalidated E values for the REF-sites correlated well with the observed (O) values (Fig. A.1). The fit between O and E was weakest for diatoms ($R^2 = 0.37$; NRMSE = 82%) and strongest ($R^2 = 0.88$; NRMSE = 52.2%), albeit somewhat more biased, for sequence-based fungi (Fig. A.1).

The model was most accurate for macroinvertebrates, with mean REF O/E_{TAXA} of 0.98. Mean O/E_{TAXA} ratios among the REF sites were slightly lower for diatoms and genus-based fungi (0.94 and 0.96, respectively; Fig. A.1) and more so (0.88) for sequence-based fungi, suggesting overestimation of expected number of native fungal OTUs. The macroinvertebrate model provided the most precise estimates of the reference condition (SD 0.17), whereas the diatom model was somewhat more imprecise (SD 0.22). Both genus- and sequence-based fungi models nearly equalled the macroinvertebrate model, SDs being 0.18 for both models (Fig. A.1). All models outcompeted the corresponding null models. The precision of the sequence-based fungal model showed the most notable improvement over the null (35%), followed by macroinvertebrate (22%), genus-based fungal (19%) and diatom model (12%).

3.2. O/E ratios in human-disturbed streams

O/E_{TAXA} ratios of macroinvertebrate assemblages did not differ from the REF sites for nutrient-enriched ($t = -0.19$; $p > 0.05$) or sediment-stressed ($t = -0.93$; $p > 0.05$) streams, whereas in acidified sites they were significantly lower ($t = -5.68$; $p < 0.001$; Fig. 4a). Furthermore, sites disturbed by both nutrients and sedimentation, or nutrients and acidification lacked a notable number of predicted taxa, resulting in mean O/E_{TAXA} ratios of 0.77 ($t = -2.30$; $p = 0.026$) and 0.46, respectively ($t = -6.17$; $p < 0.001$; Fig. 4a). Diatom O/E_{TAXA} ratios differed notably from the REF sites only for nutrient-enriched sites ($t = -4.73$; $p < 0.001$) and more marginally for the NUT + ACID sites ($t = -2.12$; $p = 0.037$; Fig. 4b).

Compared to REF sites, genus-based leaf-associated fungal assemblages had higher O/E_{TAXA} ratios in SED sites ($t = 2.93$; $p < 0.01$) and lower in NUT + SED sites ($t = -4.18$; $p < 0.001$; Fig. 4c), whereas the other stream groups did not differ from the REF sites. O/E_{TAXA} ratios of the sequence-based fungal assemblages did not differ from the REF conditions for the single-stressor sites but were lower for both multi-stressed stream groups ($t = -3.33$; $p < 0.01$ for NUT + SED and $t = -3.12$; $p < 0.01$ for NUT + ACID; Fig. 4d). Stressor interactions were mostly additive. However, NUT × SED interaction for both genus- and sequence-level fungal O/E_{TAXA} was significant ($F_{1,93} = 34.20$, $p < 0.001$; $F_{1,93} = 6.60$, $p = 0.012$, respectively), suggesting a negative synergistic response in both cases. For the genus-level data, two positives yielded a negative overall effect (a "crossover" interaction; Fig. 4c) whereas for the sequence data, two opposing individual effects produced a strongly negative overall response (Fig. 4d).

O/E ratios based on Bray-Curtis index (O/E_{BC}) were generally more sensitive in single and in particular, multiple-stressor situations. Macroinvertebrate assemblages in NUT streams differed slightly ($t = -2.60$; $p = 0.01$) from the REF sites whereas a markedly stronger deviation from

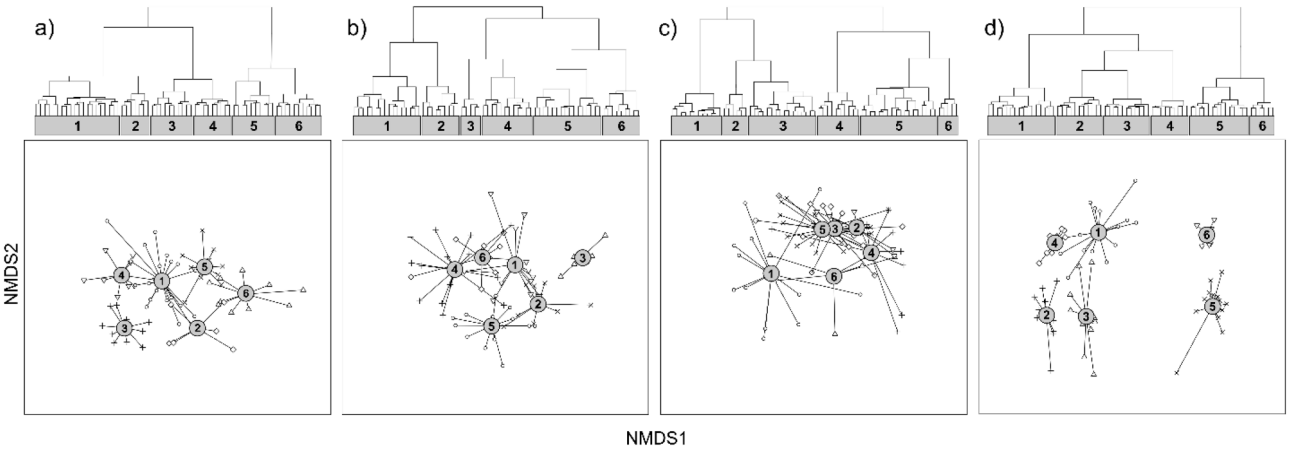

Fig 3. Agglomerative hierarchical clustering dendograms (upper panels) and NMDS ordinations (lower panels) of the 67 REF sites based on a) macroinvertebrate, b) diatom, c) genus-based fungal and d) sequence-based fungal assemblages. Grey boxes and circles in the histograms and ordinations denote the six resulting clusters, respectively.

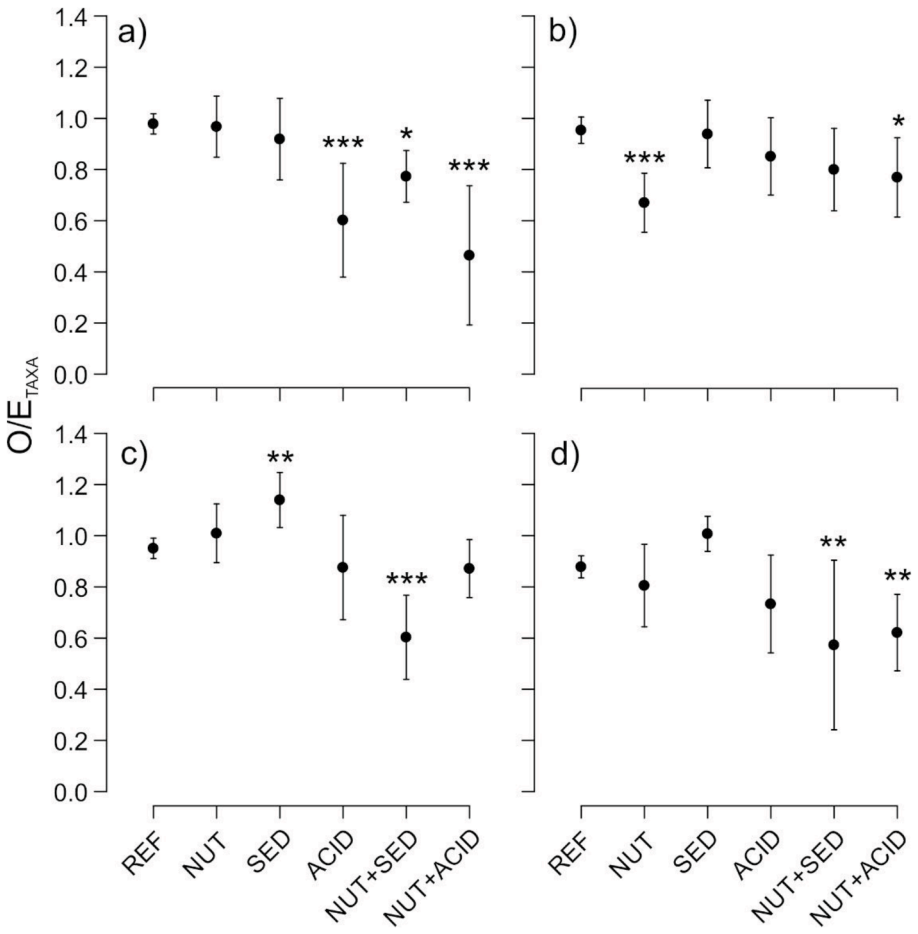

Fig. 4. Mean (±95 CI) O/E_{TAXA} ratios of a) macroinvertebrates, b) diatoms, c) genus-level fungi and d) sequence-level fungi in each stream group. All comparisons were made against the REF sites (GLM); significant differences are denoted by asterisks (*p < 0.05; **p < 0.01; ***p < 0.001). For stream-type acronyms, see Fig. 1.

the REF conditions was observed for ACID ($t = -6.91$; $p < 0.001$), NUT + SED ($t = -2.73$; $p = 0.007$) and NUT + ACID ($t = -6.48$; $p < 0.001$) streams (Fig. 5a). Diatom O/E_{BC} differed strongly from the REF sites for nutrients ($t = -6.89$; $p < 0.001$) but also for both multi-stressed stream groups ($t = -3.05$, $p < 0.01$ for NUT + SED; $t = -3.21$, $p < 0.01$ for NUT + ACID) (Fig. 5b). Genus-based fungal assemblages performed closely similarly for both O/E_{TAXA} and O/E_{BC} ratios, except for acid streams, which deviated markedly more from the REF conditions by O/E_{BC} ($t = -3.21$; $p = 0.002$) (Fig. 5c). Sequence-based fungal assemblages outperformed other taxonomic groups with O/E_{BC} index values of all disturbed stream groups differing substantially from the REF conditions (Fig. 5d). The interaction term NUT × SED was negatively synergistic only for genus-level fungi ($F_{1,93} = 20.4$, $p < 0.001$), whereas NUT × ACID interactions were significant for both genus ($F_{1,93} = 6.4$, $p = 0.01$) and sequence-level fungi ($F_{1,93} = 4.1$, $p = 0.045$). The response appeared negative antagonistic in both cases.

3.3. Leaf decomposition

Shredder-mediated leaf decomposition was significantly higher in nutrient-enriched sites ($t = 4.20$; $p < 0.001$) and lower in SED sites ($t = -2.72$; $p = 0.007$) compared to REF conditions, whereas no difference was observed for streams disturbed by acidification or by multiple simultaneous stressors (Fig. 6a). Microbial leaf decomposition differed from the REF conditions only in sediment-disturbed streams, with significantly faster decomposition in both SED ($t = 3.38$; $p = 0.001$) and NUT + SED ($t = 2.32$; $p = 0.022$) streams (Fig. 6b). NUT × SED and NUT × ACID interactions for both shredder-mediated and microbial leaf decomposition were non-significant ($p > 0.05$).

4. Discussion

Our comparison of macroinvertebrate, diatom and leaf-associated fungal assemblages revealed that aquatic fungi bear great potential for stream bioassessment based on predictive modelling. The RIVPACS-type model of fungal genera produced equally accurate and precise prediction of the reference assemblages as did the best overall model (macroinvertebrates) of the four evaluated models. This finding indicates that fungal decomposer communities can be clustered in biologically meaningful groups and this grouping can be explained, with as high a certainty as that of more traditional target groups of bioassessment, based on a few environmental attributes insensitive to human alteration.

In contrast, the sequence-based fungal model did not provide equally accurate estimates of the number of native taxa but tended to overestimate E values. This may be attributable to the massive number of fungal OTUs, which caused unwanted noise in model calculations and resulted in inaccurate estimates of the number of native taxa. On the other hand, sequence-based fungal model was superior in detecting community change in single and, even more so, multiple-stressor situations. This result implies that a species-level model should be ideal for stream bioassessment as it enables both accurate prediction of reference taxa and sensitive detection of human disturbance. Unfortunately, DNA-based species-level identification of most aquatic leaf-decomposing fungi is not yet possible.

Leaf decomposition rate showed notably high within stream-group variation, which partly prevented the detection of significant differences between the groups. However, the results clearly show that microbial and shredder-induced decomposition responded differently to human disturbance. Intensified shredder-mediated leaf decomposition

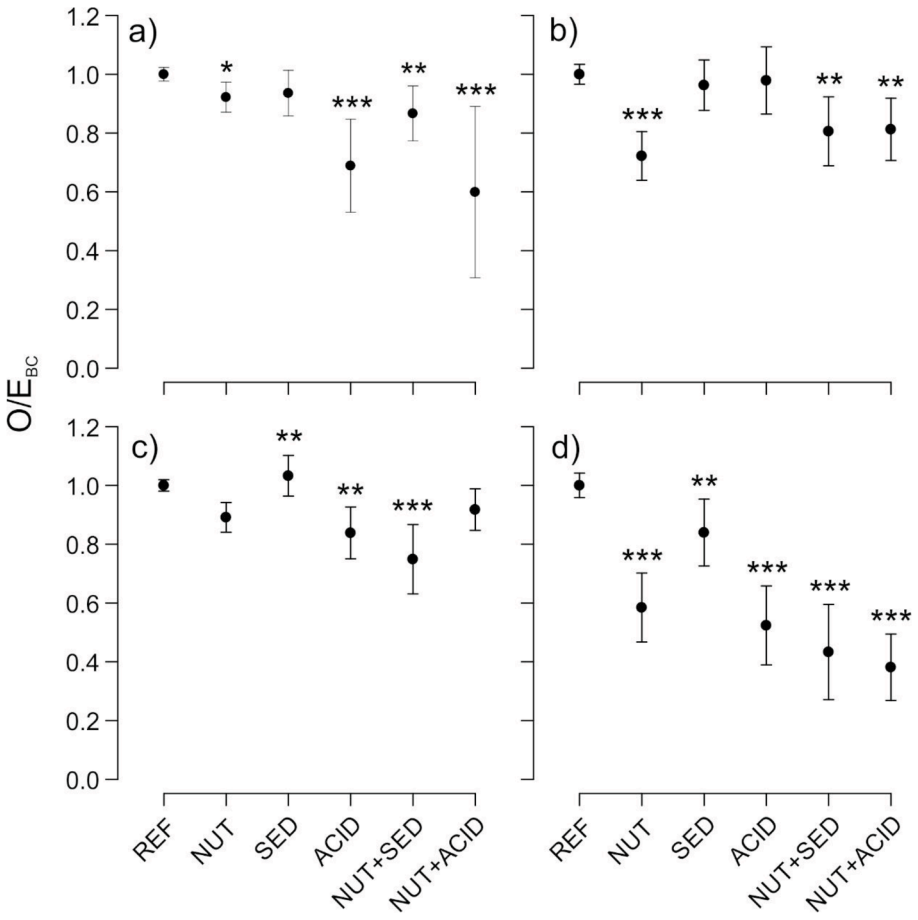

Fig. 5. Mean (±95 CI) Bray-Curtis index-based on O/E_{BC} ratios of a) macroinvertebrates, b) diatoms, c) genus-level fungi and d) sequence-level fungi in each stream group. Significant differences from the REF sites (GLM) are denoted by asterisks (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$). For stream-type acronyms, see Fig. 1.

in nutrient-enriched streams was not surprising – a similar pattern has been observed in numerous studies and has been attributed to increased microbial activity and/or shredder abundance (e.g. Robinson and Gessner, 2000; Gulis et al., 2006). In contrast, reduced shredder decomposition in sediment-disturbed streams disagrees with recent observations about the effects of sedimentation on stream ecosystem processes (Piggott et al., 2015a) and may be due to burial of the leaf bags in sand/silt -dominated streams, which may have hindered the access of macroinvertebrates into the bags. Interestingly, microbial decomposition was accelerated in sediment-disturbed streams, regardless of nutrient enrichment, a result paralleling the abrasion-induced increase of microbial decomposition rates reported by Piggott et al. (2015a).

Neither microbial nor shredder-mediated leaf decomposition showed any responses in multiple-stressor situations. For instance, the combination of nutrient enrichment (positive effect on shredder decomposition) and sedimentation (negative effect) resulted in no net change in shredder-induced decomposition. While a corresponding result has been observed in other studies (Pascoal et al., 2005b; Piggott et al., 2015a), such a lack of response in a seemingly more stressful situation may be particularly challenging to communicate to environmental managers (Piggott et al., 2015a). Even the effects of a single stressor to leaf decomposition may be hard to interpret as decomposition rate often exhibits a unimodal response to nutrient enrichment (Woodward et al., 2012; Lehosmaa et al., 2018), resulting in equal decomposition rates in low (reference) and high end of the gradient. Thus, we advocate structural components as the primary tool for stream bioassessment, especially in cases of multiple simultaneously operating anthropogenic stressors.

We detected only few interactive effects between our explanatory variables. For fungal O/E_{BC}, the interaction between nutrient enrichment and acidification was clearly antagonistic, with a combined effect that was negative but much less so than expected based on single-stressor effects. For the O/E_{TAXA} ratio, more complex interactions were observed. For the sequence-level data, nutrients and sediments alone had only a slight impact on O/E_{TAXA} ratio (negative for nutrients, positive for sediments), yet the combined effect was strongly negative. This result represents an example of an 'ecological surprise', cautioning against making management decisions based on single-stressor responses when both individual effects are minor and to opposite directions. For the same pair of stressors, O/E_{BC} of the genus-level fungal data showed 'mitigative synergism' (Piggott et al., 2015b). In this case, two positive responses yield a combined effect completely opposite to that expected based on single-stressor responses, indicating that when the two stressors operate simultaneously, they tend to mitigate their individual effects to the extent that the combined effects is less than in reference conditions ('reversal with enhancement' *sensu* Piggott et al., 2015b). From a management perspective, such combined effects are particularly challenging because they are notoriously difficult to predict based on additivity. Piggott et al. (2015b) noted that such cases of mitigating synergism are common in nature and, therefore, more research is needed to better understand which kind of stressors, and under which circumstances, are likely to produce such unexpected outcomes.

The modelling-assessment outcome for the disturbed sites was rather similar for fungi (sequence) and macroinvertebrates when O/E_{TAXA} ratios were applied. In contrast, fungal assemblages were more sensitive to both single- and multiple-stressor situations when the focus was on compositional discrepancies between the predicted and observed

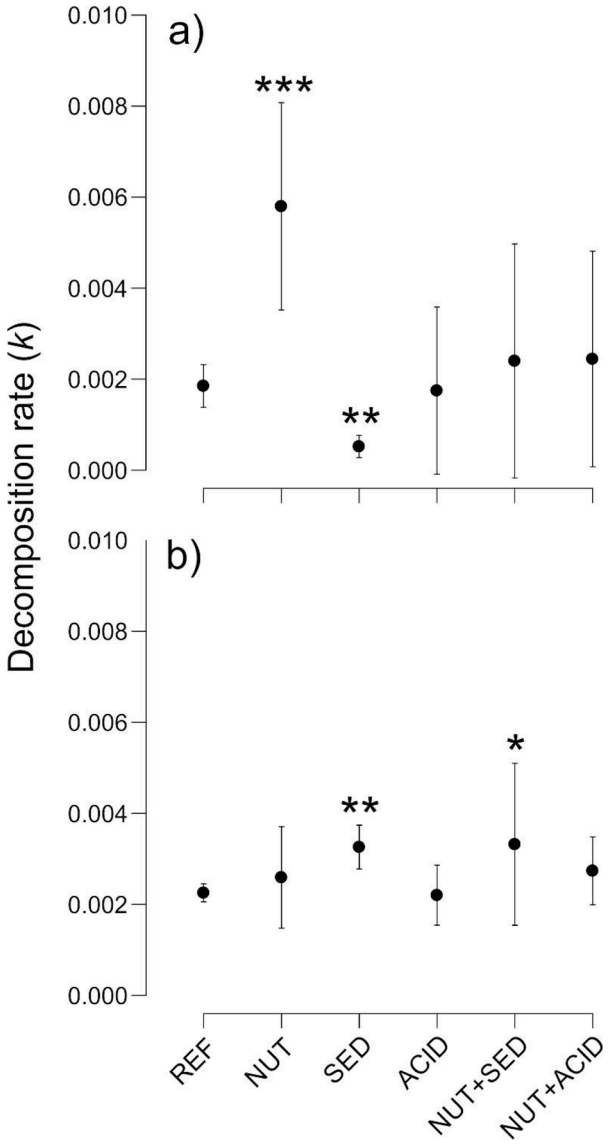

Fig. 6. Means (±95 CI) of degree days -corrected a) invertebrate (shredder) and b) microbial-induced leaf decomposition rates (k) in each stream group. Significant difference from the REF sites (GLM) are denoted by asterisks (*p < 0.05; **p < 0.01; ***p < 0.001). For stream-type acronyms, see Fig. 1.

assemblages (i.e. O/E_{BC}-ratios). In addition to focusing on compositional differences rather than pure taxa occurrence, O/E_{BC} takes all taxa into consideration, whereas the O/E_{TAXA} index emphasizes the most frequent taxa (Van Sickle et al., 2007). This result implies that the locally rare 'satellite' fungi had a major contribution to assemblage differences between the reference and disturbed sites. The role of rare taxa in stream bioassessment has been much debated but the consensus seems to be that the exclusion of rare taxa leads to improved detection of human disturbance (Poos and Jackson, 2012). A similar conclusion may not be valid for aquatic microbes, however, as some studies have suggested that rare microbial taxa may be particularly sensitive to environmental changes and their abundance/occurrence patterns correspond with the severity of disturbance (Lynch and Neufeld, 2015).

Regarding the cost-efficiency of microbial assemblages in freshwater bioassessment, it should be noted that standardized monitoring of leaf-associated fungal communities typically requires two visits – one for deployment and second for retrieving the incubated leaf material. In contrast, only single visit is needed for the traditional bioassessment elements, such as macroinvertebrates and diatoms, yet post-processing and macroscopic identification of these samples is typically laborious. However, multiple visits enable simultaneous monitoring of key ecosystem processes, such as leaf decomposition, algal accrual and microbial productivity and respiration, thereby providing more holistic perspective to the health of the stream ecosystem (Von Schiller et al., 2017).

An extremely high number of rare or low-abundance taxa is typical of microbial communities (Lynch and Neufeld, 2015). Many of these taxa remain dormant in benign environmental conditions and become resuscitated in rapidly changing or specialized circumstances, or vice versa (Lennon and Jones, 2011). In the latter case, dormancy causes substantial changes in microbial community structure by increasing the number of non-native species, hence further reinforcing taxonomic discrepancy between natural and disturbed ecosystem. Multitaxon niche model modelling has been modified recently to consider also species gains (Rose et al., 2016), which may be useful for detecting the high species turnover typical of disturbed microbial communities (Shade et al., 2012). Also focusing on active microbial communities instead of, or along with, the bulk DNA (active + inactive) using ribosomal-RNA sequencing methods (e.g. Gomez-Silvan et al., 2018), or by using RNA-based metatranscriptomics for measuring functional activities (Knight et al., 2018), could provide more information about the status and functioning of microbial assemblages and thus contribute to the improvement of microbial-based ecological assessment of stream ecosystems. Our results demonstrate that microbial assemblages have an underused potential for monitoring and assessment of freshwater ecosystems. Neither genus- nor sequence-based fungal models were ideal, however, but slightly suffered from sensitivity in detecting human disturbance and accuracy in predicting native taxa, respectively. It is therefore safe to conclude that as the costs of high-throughput sequencing are declining and the global fungal sequence libraries are progressing, the great potential of leaf-associated fungal assemblages in freshwater bioassessment, particularly that based on the reference-condition approach, can no longer be neglected.

CRediT authorship contribution statement

Jussi Jyväsjärvi: Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Software, Supervision, Validation, Visualization, Writing - original draft, Writing - review & editing. **Kaisa Lehosmaa:** Investigation, Methodology, Formal analysis, Resources, Writing - review & editing. **Jukka Aroviita:** Conceptualization, Funding acquisition, Supervision, Data curation, Project administration, Writing - review & editing. **Jarno Turunen:** Investigation, Methodology, Resources, Writing - review & editing. **Maria Rajakallio:** Investigation, Methodology, Resources. **Hannu Marttila:** Investigation, Methodology, Resources. **Mikko Tolkkinen:** Investigation, Methodology, Resources. **Heikki Mykrä:** Conceptualization, Funding acquisition, Project administration, Supervision, Writing - review & editing. **Timo Muotka:** Conceptualization, Project administration, Funding acquisition, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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